In Vitro Study of Stepwise Acquisition of rv0678 and atpE Mutations Conferring Bedaquiline Resistance

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ABSTRACT Bedaquiline resistance within Mycobacterium tuberculosis may arise through efflux-based (rv0678) or target-based (atpE) pathway mutations. M. tuberculosis mutant populations from each of five sequential steps in a passaging approach, using a pyrazinamide-resistant ATCC strain, were subjected to MIC determinations and whole-genome sequencing. Exposure to increasing bedaquiline concentrations resulted in increasing phenotypic resistance (up to $>2 \mu g/ml$) through MIC determination on solid medium (Middlebrook 7H10). rv0678 mutations were dynamic, while atpE mutations were fixed, once occurring. We present the following hypothesis for in vitro emergence of bedaquiline resistance: rv0678 mutations may be the first transient step in low-level resistance acquisition, followed by high-level resistance due to fixed atpE mutations.

KEYWORDS Mycobacterium tuberculosis, atpE, bedaquiline, in vitro model, rv0678, stepwise acquisition

Mycobacterium tuberculosis is capable of acquiring resistance to multiple drugs due to intrinsic efflux- and target-based pathways, which are key characteristics that undermine treatment success and affect morbidity and mortality.

The inclusion of bedaquiline, a novel drug, into regimens for drug-resistant tuberculosis (DR-TB) is an important step forward toward improving treatment outcomes. The early success of bedaquiline, however, is threatened by the potential emergence of resistance due to poor adherence to TB treatment, relatively weak background regimens, and the long half-life of the drug (1, 2). Interest around bedaquiline has spiked with the release of the WHO rapid communication suggesting that bedaquiline employment be prioritized and used to replace injectable drugs (3). However, bedaquiline treatment failure from clinical cases has already been reported (4, 5), with the majority of resistance due to mutations in the efflux-associated pathway. These mutations occur in the rv0678 gene encoding the MmpR repressor protein, influencing transcription of MmpL5 and MmpS5 proteins, which together make up the MmpL5-MmpS5 efflux pump (6). Alternatively, mutations in the atpE gene, the primary drug target for bedaquiline, have only been identified in two clinical isolates (5); only one of these was classified as phenotypically resistant according to the EUCAST susceptible breakpoint ($=0.25 \mu g/ml$).

To clarify the potential pathway through which M. tuberculosis bacteria acquire and evolve resistance to bedaquiline, we present a hypothesis based on in vitro generation of mutant populations.

RESULTS

The baseline solid bedaquiline MIC was 0.06 $\mu g/ml$, which is classified as sensitive, for the ATCC reference strain. Phenotypic resistance was observed after the first
passage, with solid agar MIC values of 1 μg/ml, which increased to 2 and then >2 μg/ml from the third passage onward. Similarly, the highest drug concentration permitting growth on solid medium was observed to increase from the first to the fourth passage from 0.5 to 4 μg/ml (Fig. 1).

In total, 14 distinct *rv0678* mutations but only 2 *atpE* mutations were identified in 5 passages; Fig. 1 details the passage at which they appeared. Four of the 14 *rv0678* mutations (Ile67fs, Val1Ala, Ala102Pro, and Thr33Ala) were identified in 2 passages but at low frequency. The total number of *rv0678* mutations in each population fluctuated (Fig. 1) (6→1→3→6→1) over passaging. On the other hand, a single *atpE* mutation, Glu61Asp, was maintained for four passages, while the Asp28Gly *atpE* mutation was maintained for the final two passages. In the final passage, certain mutants possessed two *atpE* mutations concurrently with *rv0678* mutations. No *atpE* mutations were detected in the first passage.

**DISCUSSION**

We present an experimental hypothesis of the underlying mutation dynamics associated with *in vitro* bedaquiline resistance acquisition in *M. tuberculosis*. Five sequential *M. tuberculosis* populations obtained through serial passaging were investigated using whole-genome sequencing (WGS). Although a variable profile of resistance-associated mutations was identified in each passage step, the phenotypic profile was consistently resistant, progressing from low to high MIC values.

*rv0678* mutations were identified in the first passage, and the number and frequency of *rv0678* mutations decrease on appearance of the Glu61Asp *atpE* mutation. Similarly, when the Asp28Gly *atpE* mutation appears at a high frequency (76%), the number and frequency of *rv0678* mutations decrease. As such, certain *rv0678* mutations may appear as precursors to high-level resistance caused by *atpE* mutations (7). Additionally, using a catalogue of *rv0678* collated from data (8), we found that the majority of the *rv0678* mutations (or positions mutated) reported in this publication (9/14) have been associated with low-level resistance either clinically or *in vitro*. We find it unlikely that an organism that is already low-level resistant would proceed to procure further mutations unless high-level resistance could be ensured.
In mutant populations with only rv0678 mutations, the solid agar MIC value was 1 μg/ml, and in populations with atpE mutations, the MIC was 2 to >2 μg/ml. Intermediate concentrations (1 to 2 μg/ml) could possibly select for both mutation types. Lower MIC values appear to be associated with rv0678 mutations and higher MIC values with atpE mutations. From the literature, we find that spontaneous bedaquiline-resistant mutants possessing only atpE mutations and either rv0678 or atpE could be selected at a 1 (8) or 0.9 μg/ml (9) concentrations, respectively. However, both these studies are performed using a spontaneous approach rather than serial passaging.

As confluent growth or an entire mutant population was scraped from plates, the frequency of mutations observed may be representative of their occurrence in the population and cannot be assigned to a single colony. The literature shows that a single atpE mutation is sufficient to result in bedaquiline resistance (9), and the same holds true for rv0678 mutations (6). While it seems unlikely that multiple rv0678 or atpE mutations would readily occur in a single organism, in this study, the Glu61Asp atpE mutation occurs at 100% alongside low-frequency rv0678 mutations, leading us to assume that every organism sequenced within this population displayed this atpE mutation and some concurrent rv0678 mutations. Using an in vitro data set comprised of clinical strains, we observed dual mutants, although this work is yet to be published (N. Ismail, personal communication). We also saw dual mutants in isoniazid-, rifampin-, and kanamycin-monoresistant strains (10). In a study by Zimenkov et al., a clinical isolate from an extensively drug resistant (XDR) patient exhibited both atpE and rv0678 mutations (5). Certain rv0678 mutations, possibly those in passages three to five, could be responsible for increased efflux pump expression due to the high drug concentration faced by the organism. Conversely, these same rv0678 mutations could be responsible for decreased efflux pump expression, as the organism has acquired atpE mutations to withstand the increased drug pressure. Further investigation of the association of different rv0678 mutations to the level of resistance is necessary to understand this phenomenon.

In this study, the Asp28Gly atpE mutation became apparent after the mutation at position 61 had occurred, initially at a 30% frequency, followed by an increase to 76% in the next passage. We postulate the appearance of this mutation could be a compensatory mutation. It is possible that if a sixth passage were investigated, the frequency could increase further as it did with the Glu61Asp mutation. While both mutations have been previously described by Segala et al. (11) to be associated with bedaquiline resistance (solid MIC, 0.5 to 1 μg/ml), they also show that mutations at position 28 exhibit greater diversity and this residue is positioned at the base of the atpE binding pocket while Glu61 is positioned at the edge of the binding pocket. Zimenkov et al. show that the Asp28Gly mutation found in clinical isolates do not confer resistance (5). While the Asp28Gly atpE mutation in vivo may play a compensatory role to increase fitness of the organism rather than be associated with resistance, further experiments investigating fitness as well as investigation of additional strains possessing this mutation may prove useful.

The hypothesis presented in this study, derived both from literature as well as observations in vitro, is depicted graphically in Fig. 2. Here, we see that a bedaquiline-resistant population may arise due to the presence of rv0678 mutations, which are either induced from bedaquiline exposure or from other drugs inducing rv0678 mutations (12). Other studies have shown that the M. tuberculosis populations that appear within lesions exhibit heterogeneity and may acquire resistance through acquisition of dynamic mutations (13, 14). Here, through the investigation of a M. tuberculosis in vitro population, we observe a population with transient rv0678 mutations with a lower MIC value than the populations possessing fixed atpE mutations (Fig. 1). These results could explain why the majority of bedaquiline-resistant clinical cases possess rv0678 mutations as opposed to atpE mutations. The effective concentration of bedaquiline inside granulomas is as yet unknown, and poor penetration could possibly explain the low number of atpE mutations observed clinically (Fig. 2) (15). Additionally, it is not possible to determine whether low-level or intermediate resistance caused by dynamic rv0678
mutations could be overcome by treating with higher concentrations of bedaquiline, as increased doses of bedaquiline impact patient cardiac health.

Although more than one of these observations may be plausible, there are limitations that must be taken into account. For extrapolation to an in vivo scenario, a key limitation is the exposure to only bedaquiline in our experimental setup compared with combination therapy in clinical practice. While suboptimal DR-TB treatment scenarios may be responsible for the appearance of \( \text{rv0678} \) mutations in bedaquiline-resistant clinical isolates, the presence of other supporting drugs may prevent the acquisition of high-level resistance in the form of \( \text{atpE} \) mutations. Other possible avenues to pursue to understand the effects of drug exposure would be to expose a mutant population to a single concentration of bedaquiline over five passages. This could clarify whether \( \text{atpE} \) mutations appear as a result of prolonged drug exposure or are driven through exposure to increasing drug concentration. Additionally, given the long half-life of bedaquiline in vivo, future experiments could be performed to observe the effect of exposure to decreasing concentrations of drug. While functional genomics studies to investigate the role of the \( \text{atpE} \) mutations in hot spot regions would be useful, an in-depth study of these mutations has been previously performed by Segala et al. (11). For \( \text{rv0678} \) mutations, previously described attempts to clone \( \text{rv0678} \) variants into integrative vectors showed partial but not complete resistance, as the wild-type \( \text{rv0678} \) gene is still expressed (6). Dynamic changes from \( \text{rv0678} \) to \( \text{atpE} \) mutations could also be further investigated through the use of a single clone as opposed to a mutant population.

We provide a hypothesis for the evolution of bedaquiline resistance through the acquisition of \( \text{rv0678} \) and \( \text{atpE} \) mutations. These findings show that \( \text{rv0678} \) mutations result in low-level bedaquiline resistance. \( \text{rv0678} \) mutations have been identified in bedaquiline resistant-clinical isolates, clofazimine-resistant clinical isolates, and in clinical isolates without prior exposure to either drug, which is overviewed in reference 15. Thus, the finding that certain \( \text{rv0678} \) mutations may behave as precursors for the appearance of \( \text{atpE} \) mutations is a cause for concern. While a number of \( \text{rv0678} \) mutations have been identified that are not associated with bedaquiline resistance (12), it would be useful to have a comprehensive catalogue of \( \text{rv0678} \) mutations with associated MICs as well as those which are linked to stepwise acquisition of \( \text{atpE} \)-based bedaquiline resistance. As mutations in the \( \text{rv0678} \) gene are scattered throughout the gene, genotypic screening of the entire gene for mutations may prove useful to identify those mutations that may negate bedaquiline usage.

**MATERIALS AND METHODS**

A pyrazinamide-monoresistant reference strain, ATCC 35828 (\( \text{pncA} \): Gly132Ser mutation), which we previously observed displaying higher baseline bedaquiline MIC values and rapidly accumulating resistance to bedaquiline compared with fully susceptible and other monoresistant strains (10), was exposed...
to increasing concentrations of bedaquiline during a serial passage approach to obtain five sequential M. tuberculosis mutant populations, as previously described (10). In brief, a McFarland standard of 1.0 cell suspension of a 21- to 28-day-old culture was used to inoculate (100 µl) four 7H10 plates. These were either drug-free (growth control) or containing bedaquiline at 0.5×, 1×, and 2× the proposed critical concentration (0.25 µg/ml). Cultures were grown at 37°C for 21 to 28 days or until sufficient growth was observed. Following this passage, confluent growth was scraped from the plate with the highest drug concentration permitting growth (Fig. 1). This growth was used for the creation of a new cell suspension for MIC determinations (below) and the following passage. Again, four plates were inoculated, namely, a drug-free control, a plate with the growth-permitting drug concentration (same as which growth was scraped from), as well as plates with 2- and 4-fold higher drug. This process was repeated until a total of five of these passages was completed.

MIC values were determined using Middlebrook 7H10 solid agar (Sigma-Aldrich), as previously described (16). In brief, cell suspensions (McFarland standard of 1.0) were prepared from actively growing cultures for baseline and mutant strains. A 10-fold dilution of this suspension was used to inoculate (100 µl) a series of bedaquiline-containing solid agar plates (range, 0.004 to 2 µg/µl). Three further dilutions were used to inoculate drug-free plates to control inoculum size and serve as positive controls for growth. Plates were incubated at 37°C for 21 days. The MIC was determined as the lowest concentration within the series with 100% visible growth inhibition.

Genomic DNA extraction was performed on the NuclISENS easyMAG platform (bioMérieux, Marcy-l’Étoile, France) using 500-µl heat-killed and bead-beaten cultured isolate, with the instrument’s generic protocol, to obtain a final eluate (25 µl) of purified nucleic acids. Preparation of paired-end libraries was performed using the Nextera XT DNA library kit (Illumina, San Diego, CA, USA), following manufacturer’s protocol for tagmentation, size selection, and modified library normalization (17). WGS was carried out using the Illumina MiSeq platform with the 2× 300-bp V3 cartridge.

Identification of rv0678 and atpE mutations was performed using the CLC Genomics workbench (version 10) and by mapping to an edited, annotated reference M. tuberculosis H37Rv genome (GenBank accession number NC_000962). The following parameters were set to identify single nucleotide polymorphisms (SNPs) or insertions/deletions (indels): forward/reverse balance of >0.015, minimum coverage depth of 5×, frequency of ≥30%, length and similarity fractions of 0.8 each, and a Phred score of ≥Q20 (≥99% accuracy) at both variant positions and nucleotides within a 5-bp radius. Additionally, using the same stringent quality measures, we reduced the frequency to 1% to observe fluctuation in rv0678 and atpE mutations.

Data availability. Raw sequence data can be found on the NCBI platform under the BioProject accession number PRJNA517607.

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We declare no conflict of interest.

REFERENCES


